SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Probes for Endoplamic Reticulum and Cell Surface Membrane Protein Dynamics.

(A-J) Probes for Endoplasmic Reticulum. (A) Single point mutations in the GluA1/2 ligand binding sites. (B) GFP fluorescence (GFP) and surface staining (surf-IR) of WT or ER-retained GFP-GluA1 and GFP-GluA2 expressed in DIV15 neurons. Scale bars, 25 μm and 15 μm (inset). (C) Relative surface expression (surface staining / total GFP fluorescence). Mean±SEM, n =13-34 cells, ***p < 0.001; t-test. (D) Hetero-oligomerization of WT and ER-GluA1/2 assessed by immunoprecipitation (IP) and immunoblotting (IB). The ~100 kDa band corresponds to HA-tagged GluA1/2 (arrow). IN, input. (E) Point mutations in VSVGts045. (F) Total GFP and surface staining of VSVGts and ER-VSVG in DIV15 neurons. Scale bar, 25 μm. (G) Surface expression of VSVGts (WT) and ER-VSVG (ER) measured as the fluorescence ratio between surface staining and total GFP at 39°C and 45 min after switching to 32°C. Mean±SEM, n = 9-32 cells; ***p < 0.001; t-test. (H) Images and quantification of Grp78/BiP immunoreactivity (BiP-IR) as a measure of the unfolded protein response. Normalized BiP-IR in non-transfected neurons (NTF) after a 24 h exposure to 2 μM thapsigargin (TG), and in neurons expressing citrine or ER-VSVG. Mean±SEM, n = 10-21 cells, 2 experiments; ***p < 0.001; t-test. Scale bar, 25μm. (I) PM-pHluorin fluorescence at the DIV15 neuronal surface at pH 7.4 or pH 5.5. Scale bar, 25μm. (J) GT46 fluorescence at the neuronal surface in DIV15 neurons. Scale bar, 10 μm.

(K) Fluorescence recovery after photobleaching (FRAP) of ER-VSVG in a neuronal dendrite (DIV15). Left, image and kymograph of ER-VSVG. Right, corresponding FRAP plot showing raw data (red) and curve fit (blue). Scale bar, 5 μm.

(L) Fluorescence along dendritic segments shown in Figure 1F at various time during repeated photobleaching at the soma. X-axis indicates proximal-to-distal length along dendrites.

(M) Similar distributions of ER-VSVG and endogenous Grp78/BiP immunoreactivity in DIV14 neuron dendrites with zones of increased ER fluorescence (arrows). Scale bar, 5 μm.

Figure S2. FRAP-Based Method to Measure ER Complexity and the Increase in ER Complexity Accompanying Dendritic Development but Unrelated to ER Entry into Dendritic Spines.

(A) Hypothetical endoplasmic reticulum (ER, green) in two dendrites with either simple (#1) or convoluted (#2) spatial organization. Blue circles indicate diffusing molecules within the ER membrane. (B) Fluorescence recovery in small (S, blue) and large (L, red) dendritic segments from #1 and #2. Dendritic regions with more complex fluorophore-accessible volumes will display a more pronounced slowing of recovery as the size of the bleached area increases. (C) Increased complexity is revealed by plotting normalized recovery rates as a function of the size of the bleached areas. Hence, by comparing the ratio of fluorescence recovery rates derived from large areas (typically >5 μm) to small areas (typically 1 μm), one obtains an index of the apparent complexity of the fluorophore-accessible volume.

(D) Fluorescence of the plasma membrane marker PM-pHluorin (top) and the ER marker ER-VSVG-YFP (bottom) in HeLa cells (pseudocolor scale). Top inset shows sequential switches to pH5.5 and quenching of the surface PM-pHluorin. Bottom inset shows reticular structure of the ER. Scale bar, 10 μm. (E) Raw (circles) and fitted (lines) recovery plots after photobleaching PM-pHluorin (blue) or ER-VSVG (red) in 1 μm (top) or 7.2 μm (bottom) regions. The slower recovery of ER-VSVG after bleaching a large region (bottom) indicates the more complex fluorophore-accessible volume of the ER relative to the PM. (F) Recovery halftimes ($t_{1/2}$) and recovery fractions (inset) as a function of the diameter of bleached areas for PM-pHluorin (blue) and ER-VSVG (red) normalized to values obtained with 1 μm bleached areas. Mean±SEM, n = 12-13 measurements for each data point over 2-3 experiments; *$p < 0.05$, **$p < 0.01$; t-test.

(G-J) Developmental increase of ER complexity. (G) ER-VSVG distribution and position of the bleached dendritic segments (1.2 - 15 μm) used for FRAP on a DIV8 hippocampal neuron. Scale bar, 5 μm. (H) Progressive slowing of ER-VSVG recovery in bleached dendrite segments of increasing length (value in white circles). (I-J) Recovery halftimes (I) and recovered fractions (J) in 1.2, 5, 10 and 15 μm long dendrite segments. Mean±SEM, n = 20, 21 cells in 4 experiments at DIV8 and DIV24, respectively; **$p < 0.01$, ***$p < 0.001$; t-test.

(K) For morphological analysis of electron micrographs, spans of serial sections defining aspiny or spiny segments of dendrites began with the first section where the base of a spine started to emerge

from the dendritic shaft and ended with the section where the base of the last spine was no longer detectable.

(L) Visualization of dendritic spines (β-actin-mCh) and ER (ER-VSVG) in hippocampal neuron dendrites. Arrows and arrowheads indicate spines containing and lacking ER, respectively. Scale bar, 5 μm. (M) Spine density does not correlate with the fraction of ER-positive spines. (N) Lack of relationship between ER complexity and ER intrusion into spines.

Figure S3. Compartmentalization of Nascent AMPA Receptors in the Dendritic ER.

(A) The spread of nascent AMPA receptors (blue) within the dendritic ER was simulated in actual dendritic tree templates assuming a constant rate of protein synthesis of ER-bound ribosomes (orange) at a dendritic branch point (red circle). See Experimental Procedures for details. Scale bar, 50 μm.

(B) ER-GluA1 density at different time points after the initiation of synthesis at the branch point marked by a red circle. The area within the white dashed box is magnified in the lower panels. See Experimental Procedures for details. Scale bars, 50 μm (upper panel) and 25 μm (lower panel).

(C) Dendritic templates with low (#1) or high (#2) degrees of branching. Scale bar, 50 μm.

(D) Fractional exploration of the total dendritic domain over time assuming constrained diffusion at branch points (BP, plain lines) or homogenous diffusion without branch point constraint (dashed lines) in the two templates (#1, #2) shown in (C). The range of AMPA receptor exploration is reduced with increasing dendritic complexity and with constrained diffusion at branch points.

(E) Simplified dendritic templates with 0 to 3 dendritic branch points distributed at equal distances from the branch point marked as the point of synthesis (red circle). Scale bar, 25 μm.

(F) Normalized ER-GluA1 concentrations in the dendritic subcompartment shown in blue in (E) after a 30 min synthesis period, as a function of branch point number. Branch points cumulatively increase receptor accumulation by diffusional confinement.

Figure S4. ER-to-Golgi Trafficking of VSVGts at Dendritic Branch Points.

(A-B) ER-released VSVGts accumulates in dendritic Golgi outposts (GOs) visualized by GFP tagged with the Golgi targeting sequence of galactosyltransferase (GT-GFP). (A) Images and time lapse. Arrowheads indicate VSVGts-mCh accumulated in a GO. (B) Quantification of VSVGts-mCh accumulation over time. Plotted is VSVGts fluorescence co-localized with GT-GFP in Golgi outposts (GO) over time, and total VSVGts fluorescence at branch points outside GO regions labeled with GT-GFP (BP outside GO). Scale bar, 5 μm. Mean±SEM, n = 13 cells; *$p < 0.05$, **$p < 0.01$; ANOVA.

(C) Immunolabeling for Sec23 after accumulation of VSVGts-mCh in GOs (same dendrite as in A). Note the complete colocalization of VSVGts with GOs (white arrowheads) and the lack of colocalization with ER exit sites labeled for Sec23 (black arrowheads). Scale bar, 2.5 μm.

Figure S5. Absence of Detectable ER Stress Upon Type I mGluR Activation by DHPG.

Phospho-PERK, Grp78/BiP, and β-actin levels (immunoblotting, pseudocolored fluorescence intensities) in DIV14 hippocampal neurons in the absence or presence of DHPG or 5 mM DTT for 1 to 5 h. In the upper blot, note the shift of PERK immunoreactivity towards higher apparent molecular mass species allowing the detection of non-phosphorylated (lower band) and phosphorylated PERK (upper band) in the presence of DTT but not DHPG. In the middle blot, note the unchanged and increased BiP expression in cells exposed to DHPG and DTT, respectively.

Figure S6. CLIMP63 Expression in Rat Hippocampus and Presence of ER in Distal Dendrites of Neurons Expressing CLIMP63 Mutants.

(A-B) Characterization of custom anti-CLIMP63 rabbit antibody. (A) CLIMP63 (green) and β-actin (red) immunoreactivity in postnatal day 7 (PND7) hippocampus detected by immunoblot using either preimmune or anti-CLIMP63 sera documenting the detection of a single band corresponding to CLIMP63. 10 or 20 μg total protein was loaded in each lane.

(B) Distribution of the ER (ER-VSVG, green) in DIV14 hippocampal neurons expressing mCherry-tagged CLIMP63-3A (3A) or 3E (3E) mutants documenting the presence of the ER in distal dendrites (arrows). Scale bar, 30 μm.

(B) Endogenous BiP immunoreactivity (green) in DIV14 hippocampal neurons documenting the presence of the ER in distal dendrites (arrows). Neurons were transduced with scrambled (Sc) or CLIMP63 (KD) shRNA with tdTomato (tdT) reporter. Scale bar, 30 μm.

Figure S7. Concentration of CLIMP63-3E at the Basis of Emerging Dendritic Branches and CLIMP63-3E Effects on AMPA Receptor Surface Expression at Synapses.

(A) CLIMP63-3E (pseudocolored) concentration at branch point (arrowheads) occurs upon new branch emergence (arrows). Outlines represent GFP expression in the emerging branch. Time in hour:min. Scale bar 10 μm.

(B-C) Representative images (B) and quantification (C) of surface GluA1 (sGluA1) immunoreactivity (IR) in DIV12 hippocampal neurons expressing the postsynaptic excitatory synapse marker homer1c-GFP (H1C) with either CLIMP63-3A or CLIMP63-3E mutants. Shown in (C) are total average (av. fluo.) sGluA1-IR in dendrites or median (med. fluo.) synaptic IR (arrowheads in B) after normalization to CLIMP63-3A values. Empty arrowheads in (B) mark extrasynaptic clusters of sGluA1. Scale bar, 5 μm. Mean±SEM, n = 13-14 cells, 2 experiments; *$p < 0.05$, **$p < 0.01$, t-test.

(D-E) Electrophysiological recordings (D) and quantification (E) of mEPSCs recorded at the soma from DIV11-DIV13 hippocampal neurons expressing CLIMP63-3A (3A) or CLIMP63-3E (3E). Note the increased mEPSC amplitude and frequency in neurons expressing CLIMP63-3E. Mean±SEM, n = 12-13 cells, 3-4 experiments for each; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; t-test.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. ER-VSVG Dynamics in Neuronal Dendrites.

Time lapse sequences of ER-VSVG fluorescence after photobleaching and comparison to typical vesicular dynamics seen with VSVGts (see Experimental Procedures). Note the smooth, uniform recovery of ER-VSVG in the photobleached segment (top) and the obvious vesicular transport into photobleached regions with VSVGts representing post-ER vesicles (bottom). Time in min:sec. Scale bars, 5 μm

Movie S2. Restricted Spatial Spread of Nascent AMPA Receptors in a Branched Dendrite.

Shown is the simulated density of GluA1 (pseudocolor scale) in the dendritic ER of a complex dendritic tree at different time points after the initiation of synthesis at the branch point marked by a red circle. Time in hour:min. Scale bar, 50 μm.

Movie S3. VAMP2-SEP Imaging Reveals Exocytosis at Branch Points.

Time lapse sequences (time in min:sec) and detection of exocytosis events (arrows) in 12-14 DIV hippocampal neurons. Branch points are defined by 5 or 2.5 μm circles centered on primary or secondary branch points, respectively. Final frames indicate several individual examples of exocytic events at branch points. Scale bar, 5 μm.

Movie S4. Reconstruction of Super-Resolution Micrographs of the Dendritic ER.

Tilt animation of three-dimensional reconstructions of the dendritic endoplasmic reticulum in hippocampal neurons expressing phospho-deficient (3A, top) and phosphomimetic (3E, bottom) CLIMP63. Scale bar, 5 μm. Note the overall simple and complex ER morphologies induced by the 3A and the 3E mutants, respectively.

SUPPLEMENTAL TABLES

Table S1. FRAP-based Analysis of VSVG and GluA1 Dynamics in the Dendritic ER.

				FRAP S (1.2 μm segment)			FRAP L (7-8 μm segment)			ER complexity
			n	$t_{1/2\,S}$ (s)	R_S (%)	α_S	$t_{1/2\,L}$ (s)	R_L (%)	α_L	$t_{1/2\,L}$ / $t_{1/2\,S}$
VSVG	proximal		18	5.7 ± 1.1	87 ± 3	0.61 ± 0.03	28.3 ± 7.5	80 ± 4	0.79 ± 0.04	5.3 ± 0.7
	distal		16	5.8 ± 1.3	82 ± 4	0.72 ± 0.06	35.7 ± 9.5	83 ± 4	0.74 ± 0.05	7 ± 1.4
	branch point		18	5.1 ± 1	87 ± 2	0.59 ± 0.05	56.5 ± 12	83 ± 5	0.76 ± 0.05	13.7 ± 2.3
VSVG	GO	w/o	10	2.6 ± 0.5	84 ± 5	0.51 ± 0.06	12 ± 4	79 ± 4	0.69 ± 0.06	4.5 ± 1
		W	10	3.6 ± 0.6	89 ± 3	0.55 ± 0.04	39.5 ± 0.6	86 ± 4	0.71 ± 0.06	13.6 ± 3.4
VSVG	NMDA	pre	34	1.8 ± 0.4	92 ± 2	0.58 ± 0.03	5.8 ± 0.1	92 ± 2	0.53 ± 0.03	3.2 ± 0.4
		post	34	1.6 ± 0.2	92 ± 2	0.52 ± 0.03	5.4 ± 0.16	90 ± 2	0.51 ± 0.02	3 ± 0.3
	DHPG	pre	42	5.2 ± 0.7	88 ± 2	0.62 ± 0.04	14.6 ± 1.6	83 ± 2	0.69 ± 0.03	3.7 ± 0.5
		post	42	4.5 ± 0.6	87 ± 1	0.62 ± 0.04	18.4 ± 2.2	86 ± 2	0.66 ± 0.03	5.5 ± 0.7
	DHPG	pre	16	2.3 ± 0.3	85 ± 4	0.64 ± 0.06	15 ± 4	84 ± 3	0.73 ± 0.05	6.7 ± 1.3
	+PKCi	post	16	4.4 ± 1	91 ± 2	0.59 ± 0.06	19.3 ± 3	85 ± 3	0.66 ± 0.05	4.9 ± 0.8
GluA1	DHPG	pre	19	18.3 ± 9.5	84 ± 3	0.79 ± 0.03	95.1 ± 19	94 ± 4	0.95 ± 0.02	11.2 ± 1.8
		post	19	8.75 ± 1.3	82 ± 2	0.72 ± 0.03	146 ± 41	97 ± 1	0.96 ± 1	17.6 ± 2.9
VSVG	+ mCh		64	3.7 ± 0.3	92 ± 1	0.59 ± 0.02	20.4 ± 2.5	85 ± 2	0.74 ± 0.02	6.1 ± 0.6
	+ CLIMP63 WT		44	3.2 ± 0.3	91 ± 1	0.53 ± 0.03	16.2 ± 2.8	86 ± 2	0.64 ± 0.03	4.3 ± 0.5
	+ CLIMP63 3A		39	2.8 ± 0.4	92 ± 2	0.57 ± 0.03	11.1 ± 2.1	89 ± 2	0.58 ± 0.03	3.5 ± 0.4
	+ CLIMP63 3E		43	7.12 ± 2.1	88 ± 2	0.69 ± 0.03	35.5 ± 4.6	85 ± 2	0.85 ± 0.02	8.4 ± 1.1

Recovery halftimes ($t_{1/2}$), fractional recovery (R), and anomalous diffusion coefficients (α) measured by FRAP in small (S, 1 μm) or large (L, 7 μm) segments of dendrites, and corresponding complexity index (ER complexity = $t_{1/2,L}$ / $t_{1/2,S}$). Abbreviations: GO, Golgi outpost; w/o, without; w, with; PKCi, PKC inhibitor (bisindolylmaleimide I, 100 nM); mCh, mCherry; VSVG, vesicular stomatitis glycoprotein; DHPG, (S)-3,5-dihydroxyphenylglycine; WT, wildtype; 3A = S3A, S17A, S19A triple alanine mutant of CLIMP63; 3E = S3E, S17E, S19E triple glutamate mutant of CLIMP63.

Table S2. Measured and Extrapolated Apparent Diffusion Coefficients (D, $\mu m^2/s$) in Straight Dendrites and at Dendritic Branch Points in DIV14-18 Hippocampal Neurons.

	ER-VSVG		ER-GluA1	
	F	D ($\mu m^2/s$)	*F*	D ($\mu m^2/s$)
Straight	1	0.151	0.44	0.067
Branch point	0.27	0.041	0.13	*0.019*

Apparent diffusion coefficients measured by FRAP in 7 μm areas and scaling factors (*F*) used for extrapolation (italics) of ER-GluA1 diffusion coefficient at branch points. Note the significant decrease in diffusion coefficient at branch points.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Constructs

GFP-tagged WT-GluA1 and GluA2 constructs (GluA1WT-PRK5-GFP and GluA2WT-PC3.1-GFP) were gifts from Dr. Richard Huganir (Johns Hopkins University). HA-tagged WT-GluA2 (pGW1-GluA2WT-HA) was a gift from Dr. Morgan Sheng (Genentech). HA-tagged WT-GluA1 were generated by cloning the PCR amplified HA open reading frame (ORF) into GluA1WT-PRK5-GFP as a MluI fragment which replaced the GFP ORF. pmCherry-N1 was derived from an mCherry encoding construct (Giepmans et al., 2006) provided by Dr. Roger Tsien (University of California, San Diego). The mCherry ORF was PCR amplified and ligated as an AgeI-BsrGI insert into the pEGFP-N1 backbone (Clontech) where it replaced GFP. VSVGts045-GFP, GT-GFP (Horton and Ehlers, 2003) and β-actin-mCherry (Helton et al., 2008) have been described previously. VSVGts045-tdT and VSVGts045-mCh were produced by replacing GFP by tdTomato or mCherry in GFP-tagged VSVGts045 construct. The GFP-SERCA2a encoding plasmid (Fukatsu et al., 2004) was a gift from Dr. K. Mikoshiba (RIKEN Brain Science Institute, Japan). The VAMP2-SEP encoding plasmid was a gift from Dr. George Augustine (Duke University).

GFP-tagged ER-GluA1 and ER-GluA2 were generated from GluA1WT-PRK5-GFP and

GluA2WT-PC3.1-GFP by site-directed mutagenesis (Stratagene) of their glutamate binding site (Figure S1A) (Greger et al., 2007; Grunwald and Kaplan, 2003), which led to a retention in the ER and diminished surface expression (Figure S1B and S1C). Receptors assembled from these subunits were still able to hetero-oligomerize (Figure S1D), demonstrating that ER-retention was not due to a failure to initiate folding and assembly.

GFP tagged ER-VSVG was generated from VSVGts045-GFP constructs (Horton and Ehlers, 2003) by site-directed mutagenesis of VSVGts45 COPII binding domains (Nishimura and Balch, 1997; Sevier et al., 2000) (Figure S1E), which led to constitutive retention in the ER (Figure S1F and S1G). Photoactivatable ER-VSVG (ER-VSVG-paGFP) was produced by replacing GFP by paGFP (Patterson and Lippincott-Schwartz, 2002) in the GFP-tagged ER-VSVG construct. PM-pHluorin (PM-pHluo) was generated by PCR amplification of the superecliptic pHluorin (Miesenbock et al., 1998; Sankaranarayanan et al., 2000) from a plasmid provided by Dr. George Augustine (Duke University), and cloned as a BglII-SalI insert downstream and in frame with the signal peptide sequence of the cell-surface expression type1 membrane protein backbone pDisplay (Invitrogen). The YFP-tagged GT46 encoding plasmid (Kenworthy et al., 2004; Pralle et al., 2000) was a gift from Dr. S. Grinstein (University of Toronto).

CLIMP63-WT, CLIMP63-S3A, S17A, S19A (3A), CLIMP63-S3E, S17E, S19E (3E) (Vedrenne et al., 2005) constructs were gifts from Dr. H. P. Hauri (University of Basel, Switzerland). mCherry-tagged constructs were generated by PCR amplification of the CLIMP63 ORF as a XhoI-EcoRI fragment inserted into pmCherry-N1. For expression in neurons, the CMV promoter of these plasmids was attenuated as described (Hanus et al., 2006) and all constructs were verified by sequencing. GFP-homer1C construct was a gift from Dr. Daniel Choquet (Université de Bordeaux, France).

shRNA Knockdown of CLIMP63

For shRNA knockdown of CLIMP63, we used the following target sequence: UCAACCGUAUUAGUGAAGUUCUACA. The DNA oligonucleotides containing the shRNA target sequence, a 10 nucleotide loop region (*TTGATATCCG*), and the shRNA antisense

sequence were ligated and cloned into pLentilox3.7 (a gift from Dr. Tyler Jacks, MIT) (Kennedy et al., 2010) modified to express tdTomato as a cell fill under a synapsin I promotor. pLentilox3.7 expressing a scrambled shRNA sequence was used as a negative control. The efficacy of our shRNA sequences was assessed by immunocytochemistry and immunoblotting in hippocampal neurons transduced with pLentilox3.7 harboring either scrambled control shRNA or shRNA targeting CLIMP63, and resulted in a 65% reduction of CLIMP63 levels. For FRAP experiments, CLIMP63 shRNA and scrambled control constructs were transfected and expressed from DIV2 to DIV11. To minimize ER stress, ER-VSVG was introduced at DIV10 by a second transfection and imaged 12-24 h post-transfection. To monitor dendritic morphogenesis at DIV11, neurons were transduced with lentivirus expressing tdTomato upon plating with subsequent transfection of shRNA-resistant CLIMP63-3A at DIV5.

Antibodies

The following antibodies were used for immunocytochemistry (ICC), immunoprecipitation (IP), or immunoblotting (IB) at the indicated concentration/dilution. Mouse anti-VSVG (Horton and Ehlers, 2003) (ICC, 1/300), rabbit anti-GFP (Clontech, IP, 1 μg/mL), chicken anti-GFP (Chemicon, ICC, 1:1000), mouse anti-HA (Roche, IB, 1:1000), rabbit anti-Grp78/BiP (Abcam, ICC, 1:300; IB, 1:2000), rabbit anti-Sec23 (Abcam, ICC, 1:300), mouse anti-GM130 (BD Bioscience, ICC, 1:750), rabbit anti-PERK (Cell Signaling, IB, 1:1000), mouse anti-β-actin (Sigma, IB, 1:10,000), rabbit anti-RFP (Rockland, IB, 1:1000), mouse anti-MAP2 (Millipore, ICC, 1:2000), rabbit anti-GluA1 ((Kennedy et al., 2010), ICC, 1:300), mouse anti-GluA2 (Chemicon, ICC, 1:300), goat rhodamine-RX or FITC-conjugated anti-IgG secondary antibodies (Jackson Laboratory, ICC, 0.75 μg/mL), goat Alexa 647-conjugated secondary antibodies (Molecular Probes, ICC, 1:400), HRP-conjugated (Amersham, IB) and IRDye secondary antibodies (Li-cor, IB, 1:15,000) .

A custom anti-CLIMP63 rabbit polyclonal antibody was raised (Open Biosystems) against rat CLIMP63 amino acids 14-27 (peptide CHGAASP(pS)DKGAHPSG, Open Biosystems) and characterized as described in Figure S6A. Unchanged antibody recognition of wildtype CLIMP63 upon increased (2 μM PMA, Tocris) or decreased (lambda phosphatase)

phosphorylation and equivalent recognition of CLIMP63-S19A demonstrated that this antibody was not phosphospecific and could be used as a pan-CLIMP63 antibody (data not shown).

Immunoprecipitation and Immunoblotting

Immunoprecipitation experiments were performed in 293T cells. Cells were scraped in PBS supplemented with protease (Roche) and phosphatase (Sigma) inhibitor cocktails and disrupted by sonication. Membrane fractions were collected by ultracentrifugation for 30 min at 100,000 x g (Beckman Coulter Optima) and solubilized overnight at 4°C in RIPA buffer (150 mM NaCl, 25 mM Tris, 1% sodium deoxycholate, 0.1% SDS and 1% NP40, pH 7.6) supplemented with protease (Roche) and phosphatase inhibitor cocktails (Sigma). Proteins of interest were co-immunoprecipitated by sequential incubation with 1 µg rabbit anti-GFP for 2 h and protein G-coated sepharose beads (GE Healthcare) for 1 h at 4ºC.

Cultured hippocampal (Figures 7G and S5) neurons were directly collected in RIPA buffer supplemented with benzonase (Sigma) and protease inhibitors (Roche) in the presence or, for subsequent λ phosphatase treatment, in the absence of phosphatase inhibitor cocktail (Sigma). For analysis of brain tissue (Figure S6A), postnatal day 7 hippocampi were dissected in Gey's balanced saline solution supplemented with 6.5% glucose and homogenized in 4 mM HEPES and 1.32 M sucrose, pH 7.4, and then lysed for 1 h at 4°C with 0.3% SDS in the presence of benzonase (Sigma). Lysates were resolved by SDS-PAGE in 4-12% Bis-Tris gels (Invitrogen) and analyzed by immunoblotting using chemiluminescence (ECL Plus, GE Healthcare) or near infrared fluorescence (Li-cor Lifesciences Inc.).

Immunocytochemistry

Cells were fixed for 15 min at RT in 4% paraformaldehyde/PBS (Serva, Germany) and then incubated for 30 min in 5% BSA/PBS (blocking solution) after 15 min permeabilization in 0.15% (w/v) Triton-X100/PBS. Incubations with primary and secondary antibodies were performed in 1.5% BSA/PBS either overnight at 4ºC or for 1 h at RT. Coverslips were mounted on glass slides in ImmuMount medium (Thermo).

Sec23 immunolabeling was performed with a separate protocol (Hammond and Glick, 2000; Horton and Ehlers, 2003). In brief, cells were first fixed in ice-cold methanol for 3 min, rehydrated in 0.1% N-octyl-beta-D-glucopyranoside incubated (OGP) and 100 μM of the cross linker bis-sulfosuccinimidyl for 30 min at RT and incubated in 0.1% ethylenediamine-HCl pH 7.5 for 15 min and blocked in the presence of OGP in 3% fish gelatin.

For labeling of surface AMPA receptors, neurons were incubated with primary antibodies for 12 min at room temperature before fixation and were then processed as described above.

Image Acquisition and Analysis

Confocal imaging was performed using a 60x 1.4 NA objective on a TE300 Nikon inverted microscope equipped with a CSU10 spinning disk confocal unit (Yokugawa, Inc.), an EM-CCD camera (Hamamatsu, Inc.), and a custom 405, 488, 568 and 640 nm diode-laser illumination module (Prairie Technologies, Inc.). Photobleaching and photoactivation experiments were performed using a 63x 1.4 NA objective on a Zeiss LSM5-LIVE inverted confocal microscope, with the pinhole open at 5-6 Airy units and images acquired using 2 x 2 pixel binning. For long-term imaging experiments (12-24 h), neurons were imaged in conditioned Neurobasal/B27 medium (Invitrogen) supplemented with 7.5 mM HEPES in a 5% CO_2 atmosphere using a 20x 0.8 NA objective on a Zeiss LSM5-LIVE inverted confocal microscope. Live cell imaging experiments were performed at 32°C (VSVGts) or 37°C. All image analysis was performed using Metamorph software (Universal Imaging Corporation).

Variation of ER fluorescence and Sec23 levels along dendrites. Integrated fluorescence intensities were measured after application of a 3 x 3 matrix low pass filter and background correction. To exclude ER fluorescence variation due to local heterogeneity of dendritic shapes, ER fluorescence variation coefficients throughout dendrites (standard deviation / mean) were normalized to the pixel-to-pixel fluorescence correlation index measured for GFP and a red cell fill (mCherry). Sec23 immunoreactivity was measured in segments of dendrites defined by 5 and 10 μm radius circles centered at branch points. Enrichment at branch point was quantified as the ratio of average fluorescence in the center 5 μm segment to the average fluorescence in the two 2.5 μm long flanking segments.

VSVGts accumulation in Golgi outposts at dendritic branch points. Z-stacks of GT-GFP and VSVGts-mChy were acquired at 5 min (for early time points, 10-30 min after 39-32°C temperature shift) or 10 min (for later time points, 30-40 min after temperature shift) intervals. VSVGts-mCh fluorescence was quantified at branch points displaying GOs in the first 200 µm apical dendrite of hippocampal neurons using maximal projections. Regions of interest were traced by thresholding (GOs) or manually (BPs). BP areas were defined as sections of dendrites included in 4 µm circles centered on GOs. VSVGts-mCh accumulation was measured as the integrated fluorescence intensity inside or outside outlines of GOs within BP areas after application of a 3 x 3 matrix low pass filter and background correction.

VAMP2 exocytosis. Time-lapse sequences (101 frames) of VAMP2-SEP were acquired in single focal planes at 1s intervals every 10 min starting 10 min after 39-32°C temperature shift. Exocytosis events were identified as one step increase of SEP fluorescence and were counted in the first 200 µm proximal portions of primary and secondary dendrites that were in focus. Dendritic surfaces used to calculate event density ($N/\mu m^2$) were traced manually. Branch points were defined as described above in 5 µm or 2.5 µm circles centered on large or smaller branch points, respectively.

Dendritic branch formation and zones of ER complexity. The number of new dendritic branches emerging from zones of ER-complexity was quantified in the first 200 µm of apical dendrites. Zones of ER complexity were defined by local enrichment of CLIMP63-3E-mCh over periods of at least 5 hours and were only considered if initially localized in straight dendritic segments. Only dendritic protrusions longer than 10 µm and maintained for at least 5 hours were quantified as newly formed branches.

AMPA receptor surface expression and synaptic levels. Surface GluA1-IR was quantified in the first 150 µm of the apical dendrite of pyramid-shaped hippocampal neurons. Dendrites were manually traced on the overlaid images of GFP-homer1C and GluA1-IR. Only segments where synapses could be assigned to the cell of interest were included in the analysis. Surface expression was quantified as the average fluorescence measured on the entire dendrite after application of a low pass filter and background correction. For analysis of synaptic receptor clusters, 0.8 µm circles (9 pixels) were centered on excitatory postsynaptic densities marked by

homer1c-GFP and on GluA1 clusters. Synaptic clusters were defined as GluA1 clusters overlapping with homer1c-GFP (overlap of 10 pixels or more).

ER entry into spines. The low fluorescence of ER membranes in dendritic spines and the relatively low spatial resolution and high sensitivity to photobleaching of FRAP experiments did not allow for quantification of ER-VSVG mobility and ER entry into spines in the same cells. These quantifications were thus done in parallel in live cells (FRAP) and after fixation. In live cells, spines were visualized by expressing a red cell fill (mCherry) and only spiny neurons displaying with clear mushroom spines were analyzed (Wang et al., 2008) (Figure 2F). Spine density was calculated by counting protrusions regardless of their morphology (stubby, mushroom-like, etc.) (Spacek and Harris, 1997) in 10 μm dendritic segments centered on photobleached areas. In fixed cells, spines were visualized by expressing β-actin-mCherry (Figure S2L), imaged at high resolution, and counted in 50-100 μm dendritic segments. The clear visualization of ER entry into spines required a saturation of ER-VSVG-GFP signal in the dendritic shaft, and was quantified manually on an all-or-none basis using a constant fluorescence scaling. Variation of ER complexity as a function of ER entry into spines was determined by calculating partial averages of these two parameters after binning the samples in groups of increasing spine density (0.1 spine / μm increments).

FRAP Analysis

The time required for photobleaching was <0.5 sec and was negligible compared to the duration of recovery. Only cells displaying >70% photobleaching relative to initial fluorescence in the bleached area and a global photobleaching during image acquisition <15% were used for analysis. All data were corrected for photobleaching during image acquisition and processed using Matlab (The MathWorks Inc.). Photobleaching was performed on 4μm long dendritic area (Figure 1D, 1E and S1K) or otherwise specified in the Results. Experiments in neuronal dendrites were performed in dendritic segments of matched diameters at similar distances from the cell body.

FRAP in fibroblasts. The normalized fluorescence intensity F(t) in bleached areas at time t was analysed using the two-dimensional diffusion model as described (Feder et al., 1996) and fitted with the equation:

$$F(t) = \frac{F_1 + (R(F_0 - F_1) + F_1)\left(\frac{t}{t_{1/2}}\right)^{\alpha}}{1 + \left(\frac{t}{t_{1/2}}\right)^{\alpha}} \qquad (1)$$

where F_0 is the fluorescence intensity before bleaching, F_1 is the fluorescence intensity immediately after bleaching, R is the recovered fluorescence fraction, and $t_{1/2}$ is the recovery halftime.

FRAP in neuronal dendrites. A custom fitting model was used for analysis according to Fick's second diffusion law (Crank, 1975). Assuming three-dimensional diffusion in cylindrical structures, the concentration C of fluorophore at position **r** within the photobleached segment at time t, C(**r**, t), along with the concentration gradient ∇ induced by photobleaching are related by Fick's second diffusion law written as:

$$\frac{\partial C(\mathbf{r},t)}{\partial t} = D(t)\nabla^2 C(\mathbf{r},t) \qquad (2)$$

The diffusion was considered to be potentially anomalous, implying a diffusion coefficient D varying with the transport coefficient Γ such that

$$D(t) = \frac{1}{4}\Gamma t^{\alpha-1} = D_1 t^{\alpha-1} \qquad (3)$$

with **r** included in the photobleached rectangular profile of length 2*w*, a symmetric recovery at each side of the bleached cylinder, and an absence of diffusion in the direction *n* perpendicular to its axis. The boundary conditions were:

$$\begin{aligned} &C(w,\ t) = C(-w,\ t) = C_0, t \geq 0, \\ &C(\mathbf{r},t) = 0, t = 0, \\ &\left.\frac{\partial C(\mathbf{r},t)}{\partial n}\right|_{\Omega} = 0 \end{aligned} \qquad (4)$$

Dendrites were considered to be cylinders along which C(**r**, t) is equal in the plane orthogonal to

the cylinder axis at position **r**. The equation (2) can then be approximated using a one-dimensional diffusion relationship where the second Fick's law becomes

$$\frac{\partial C(x,t)}{\partial t} = D(t)\frac{\partial^2 C(x,t)}{\partial x^2}, \qquad (5)$$

with *x* being the distance along dendrites from the center of the photobleached rectangle. With the symmetry present at $x = 0$, boundary conditions can be written in the form:

$$\begin{aligned} &C(w, t) = C(-w, t) = C_0, t \geq 0, \\ &\frac{\partial C(x,t)}{\partial x} = 0, x = 0, t \geq 0 \\ &C(x,t) = 0, t = 0, |x| < w \end{aligned} \qquad (6)$$

The differential equation (6) was solved using the method of separation of variables and integration by parts, resulting in an equation of the form:

$$C(x,t) = C_0 - \frac{4C_0}{\pi}\sum_{n=0}^{+\infty}\frac{(-1)^n}{2n+1}\exp\left(-\frac{D_1(2n+1)^2\pi^2 t^{\alpha}}{4\alpha w^2}\right)\cos\frac{(2n+1)\pi x}{2w} \qquad (7)$$

The normalized fluorescence intensity F(t) observed at time ≥0 and calculated in the entire bleached rectangle could thus be expressed in the form:

$$F(t) = R\left(1 - \frac{8}{\pi^2}\sum_{n=0}^{+\infty}\frac{1}{(2n+1)^2}\exp\left(-\frac{D_1(2n+1)^2\pi^2 t^{\alpha}}{4\alpha w^2}\right)\right) \qquad (8)$$

with R the recovered fraction, D_1 the apparent diffusion coefficient, α an "anomalous" coefficient, and $t_{1/2}$ the recovery halftime, which were calculated by minimization of the mean-squared error. The sum used for the integration was truncated with *n* varying from 0 to a nonnegative integer N (chosen as 10 across the analysis). Throughout the present study, calculated α coefficients were not significantly affected under any of our experimental conditions (Table S1).

The quality of FRAP plot fitting was estimated by R-square values (Bevington, 1992). Only plots showing fitting within 90% statistical confidence were included in our analysis (>90% of the neurons we monitored).

Modeling Nascent AMPA Receptor Diffusion in the Dendritic ER

Synthesis rates and accumulation of nascent GluA1 and GluA2. Since ER-bound ribosomes (Figure 4A) and Golgi outposts (Horton et al., 2005) concentrate at branch points, we selected branch points as points of origin of newly synthesized AMPA receptors. However, the model can be applied to any arbitrary point source or multiple point sources along the dendrite. Due to the combined effect of translation initiation, elongation, protein folding, and subunit assembly, it is difficult to estimate realistic synthesis rates of a given membrane protein *a priori* (Hershey, 1991). Rough estimates of nascent GluA1 and GluA2 accumulation rates were extrapolated from protein elongation rates measured *in vitro* (~6 amino acids/second per active ribosome) (Hershey, 1991), the estimated number of ER-bound ribosomes present at dendritic branch points in our EM micrographs, the relative abundance of GluA1 and GluA2 mRNA in hippocampal neuron dendrites reported in the literature (Grooms et al., 2006; Poon et al., 2006; Tsuzuki et al., 2001), and the relative abundance of secreted and transmembrane proteins found in the murine transcriptome (http://locate.imb.uq.edu.au/) (Sprenger et al., 2008). Single cell PCR measurement indicate that GluA1 and GluA2 mRNAs represent approximately 1/240 and 1/500 of total cellular mRNAs in cultured hippocampal pyramidal neurons, respectively (Tsuzuki et al., 2001). Assuming that GluA2 mRNA displays the same relative dendritic enrichment as GluA1 mRNA (~3.2) (Grooms et al., 2006; Poon et al., 2006), GluA1 and GluA2 encoding mRNAs can be expected to represent ~1/75 and ~1/150 of total dendritic mRNAs, respectively. Among the 58,128 protein sequences present in the RIKEN FANTOM mouse protein sequence database (Carninci et al., 2005), 4231 correspond to secreted (signal-peptide containing) proteins, and 14,088 correspond to membrane (transmembrane domain containing) proteins (http://locate.imb.uq.edu.au/) (Sprenger et al., 2008). Although this estimation does not take into account mitochondrial transmembrane proteins, cytoplasmic proteins processed by ER-bound ribosomes, or neuron-specific transcriptomes, one can estimate that ~31.5% of mRNAs will be processed by ER-bound ribosomes in a generic murine cell. Assuming that mRNA dendritic enrichment preserves these proportions, one can estimate that GluA1 and GluA2 mRNAs represent ~1/23.6 and ~1/47.2 of dendritic mRNAs processed by ER-bound ribosomes, respectively. Finally, assuming optimal and equal translation initiation rates and an average size of 700 codons for all dendritic mRNAs, the ~100 ER-bound ribosomes found at a single generic dendritic branch point could in principle assemble 600 amino acids per second, thus 51 proteins

and therefore ~2.2 GluA1 and ~1.1 GluA2 per minute. The potential effects of their association with ER chaperon proteins, post-translational modifications, and ER associated degradation (ERAD) on nascent receptor diffusion were not considered in the present model.

Computation of nascent receptor densities along dendrites. Reference dendritic trees were obtained by imaging DIV24 hippocampal neurons expressing a red cell fill. Dendritic trees were segmented into 7 μm long segments (the sized of bleached area used for FRAP) starting at dendritic branch points. Apparent diffusion coefficients estimated for branch points or straight spiny dendrites (Table S2) were assigned to the resulting structures. The density of nascent GluA1 and GluA2 produced at the rates described above at a randomly chosen dendritic branch point over time was then computed in Matlab using a modified version of equation (7), allowing the incorporation of distinct apparent diffusion coefficients assigned to specific dendritic regions. Shown in Figure S3 and Movie S2 are color-coded densities of nascent GluA1 computed at different time points.

For the analysis, equation (5) was modified so that D became a function of the distance x along the dendritic tree from the origin. With x_s and R_s being the coordinate of the origin branch point and associated protein synthesis rate, respectively, the diffusion within a dendritic branch *i*, was expressed in the form:

$$\frac{\partial C_i(x_i,t)}{\partial t} = \begin{cases} D(x_i)\dfrac{\partial^2 C_i(x_i,t)}{\partial x_i^2} + R_s, & \text{if } x_i = x_s, \\ D(x_i)\dfrac{\partial^2 C_i(x_i,t)}{\partial x_i^2}, & \text{otherwise.} \end{cases} \qquad (9)$$

Assuming that dendritic branches are narrow enough to be considered as elastic tubes along which diffusion rate and protein density is constant at any arbitrarily small cross section, protein entry into dendritic branch points upon bifurcation will be directly proportional to the cross sectional area of each branch. For example, considering protein flow entering branches 1 and 2 from branch 0, with $F_i(x_b)$ i = 0, 1, 2 the transfer rate per area unit, D_i the diffusion coefficient at the branch point x_b, and r_i the radius of branch *i*, mass conservation at branch points implies that:

$$\pi r_0^2 F_0 = \pi r_1^2 F_1 + \pi r_2^2 F_2 \Rightarrow r_0^2 D_0 \frac{\partial C_0(x_b,t)}{\partial t} = r_1^2 D_1 \frac{\partial C_1(x_b,t)}{\partial t} + r_2^2 D_2 \frac{\partial C_2(x_b,t)}{\partial t}. \qquad (10)$$

With T and B the two sets encompassing all dendritic ends and branch points, respectively, nascent protein diffusion throughout the entire dendritic tree can be described using the following partial differential equations:

$$\begin{aligned}
\frac{\partial C_i(x_i,t)}{\partial t} &= \begin{cases} D(x_i)\dfrac{\partial^2 C_i(x_i,t)}{\partial x_i^2} + R_s, & \text{if } x_i = x_s, \\ D(x_i)\dfrac{\partial C_i(x_i,t)}{\partial x_i^2}, & \text{otherwise.} \end{cases} \\
r_0^2 D_0 \frac{\partial C_0(x_b,t)}{\partial t} &= r_1^2 D_1 \frac{\partial C_1(x_b,t)}{\partial t} + r_2^2 D_2 \frac{\partial C_2(x_b,t)}{\partial t}, \forall x_b \in \mathrm{B} \\
\frac{\partial C_i(x_e,t)}{\partial x} &= 0, \forall x_e \in \mathrm{T} \\
C_i(x_i,0) &= 0, \forall x_i.
\end{aligned} \tag{11}$$

Unlike FRAP data analysis where closed form solutions could be derived, it was difficult to obtain analytical solution of (11). We thus used a finite difference method to solve (11). At time t, we could approximate:

$$\frac{\partial^2 C_i(x_i,t)}{\partial x_i^2} \approx \frac{C_i(x_i-\Delta x,t) - 2C_i(x_i,t) + C_i(x_i+\Delta x,t)}{\Delta x^2}, \tag{12}$$

where $\Delta x > 0$ is a small step size. We then updated $C_i(x_i, t+\Delta t)$ using the relations:

$$\begin{aligned}
C_i(x_i,t+\Delta t) &\approx C_i(x_i,t) + \frac{\partial^2 C_i(x_i,t)}{\Delta x_i^2}\Delta t \\
&\approx C_i(x_i,t) + \frac{C_i(x_i-\Delta x,t) - 2C_i(x_i,t) + C_i(x_i+\Delta x,t)}{\Delta x^2}\Delta t, \quad \text{if } x_i \neq x_s,
\end{aligned} \tag{13}$$

where Δt is a small time step size. When $x_i = x_s$, we only needed to add $R_s \Delta t$ to the right hand side of (13). At the branch point x_b, we thus have

$$\begin{aligned}
& r_0^2 D_0 \frac{C_0(x_b,t+\Delta t) - C_0(x_b-\Delta x,t+\Delta t)}{\Delta x} \\
&= r_1^2 D_1 \frac{C_1(x_b+\Delta x,t+\Delta t) - C_1(x_b,t+\Delta t)}{\Delta x} + r_2^2 D_2 \frac{C_2(x_b+\Delta x,t+\Delta t) - C_2(x_b,t+\Delta t)}{\Delta x}
\end{aligned} \tag{14}$$

Note that $C_0(x_b,t+\Delta t) = C_1(x_b,t+\Delta t) = C_2(x_b,t+\Delta t)$. Therefore,

$$C_0(x_b,t+\Delta t) = \frac{r_0^2 D_0 C_0(x_b-\Delta x,t+\Delta t) + r_1^2 D_1 C_1(x_b+\Delta x,t+\Delta t) + r_2^2 D_2 C_2(x_b+\Delta x,t+\Delta t)}{r_0^2 D_0 + r_1^2 D_1 + r_2^2 D_2} \tag{15}$$

Finally, the third boundary condition in (11) implies that:

$$C_i(x_e, t+\Delta t) = C_i(x_e - \Delta x, t+\Delta t), \forall x_e \in \mathrm{T}.$$

By executing (13)-(15) in Matlab, we obtained the numerical solution of (11).

Electrophysiology

Whole cell voltage clamp recordings were performed in DIV10-13 neurons after expressing mCherry and CLIMP63 phosphomutants for 3-6 days. Neurons were held at -60 mV using a MultiClamp 700A amplifier (Axon Instruments, CA) controlled by a Pentium PC running MultiClamp Commander and pClamp (Axon Instruments) in extracellular solution containing (in mM) 150 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, 30 D-glucose, 2 $CaCl_2$, 0.001 TTX and 0.03 bicuculline (330 mOsm/l, pH 7.4). Recording pipettes, with resistances between 3-5 MΩ, were filled with a solution containing (in mM) 30 $CsSO_4$, 70 K_2SO_4, 25 HEPES, 25 N-methyl-D-glucamine, 0.1 $CaCl_2$, 1 EGTA, 2 Na_2ATP and 0.1 leupeptin (300 mOsm/l, pH 7.2). Data were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA). mEPSC traces with amplitude greater than 5 pA and rise times (from the onset to the peak) less than 5 ms were included in the analysis.

Statistics

Throughout the paper, data are presented as means ± SEM. The number of cells and independent experiments used for quantifications are indicated in the text or in the figure legends. Statistical significance was determined using 2-tailed unpaired or paired t-tests when comparing paired sets of data obtained in cell populations or in individual cells, respectively. ANOVA was used when simultaneously comparing more than two data sets.

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